PROJECT REPORT

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DETECTION OF SOIL MICROORGANISMS IN SITU BY COMBINED GAS CHROMATOGRAPHY-MASS SPECTROMETRY

INTRODUCTION

The research period was used to study the nature and quantity of volatile organic microbial metabolites generated in terrestrial soils. Laboratory experiments, carried out under anaerobic conditions with addition of carbon source, have been extended to include a variety of soils and additional substrates. In situ experiments were conducted without amendment using a vacuum sampling system.

OBJECTIVES

The major objective is to determine whether analysis of volatile metabolic products, formed in situ, is a viable procedure for an extraterrestrial life detection system. Information on the kinds and amounts of volatile products generated in terrestrial soils, and the parameters that influence their formation and detection, is essential to the development of the in situ procedure.

EXPERIMENTAL METHODS

Analytical Methods:

Volatile metabolites were trapped and analyzed by combined g.c.-m.s. as previously described. For quantitative determination of metabolites the ion monitor signal was recorded to give a "chromatogram", and peak areas for each component were compared with standard curves.

Carbon dioxide was determined by titration after absorption in standard base.

Microbial populations were determined by the plate count method following anaerobic incubation in soil extract agar.

Laboratory Experiments:

The dynamic system described in the 1970-71 report was used for all experiments. The only change to the system was the addition of an Oxy-sorb column to remove trace amounts of oxygen from the argon gas. All soils were amended with water to 1/3 bar. The three general substrate amendments used during the reporting period were:

(i) glucose at 1% w/w (air dry soil).

(ii) glucose and either peptone, or individual amino acids at 1% and 0.5% w/w, respectively.

(iii) amino acid mixture consisting of equal weights of lysine, ornithine, histidine, tyrosine and glutamic acid at 1%.

In Situ Experiments:

A one inch core was removed from the soil and the soil probe inserted (see 1970-71 report). The soil atmosphere was drawn through a poropak trap, using a vacuum pump, and the contents of the trap analyzed at appropriate intervals. The vacuum pump was a diaphragm aquarium pump, modified to operate off a 12 VDC automobile battery. One pump would operate for 30 days on a 72 amp. hour battery. A needle valve was used to regulate the sampling rate, which was measured periodically with a flow meter. The sampling system is shown diagramatically in figure 13.

RESULTS AND DISCUSSION

LABORATORY STUDIES

a) Soils amended with glucose

The study of volatile metabolites generated in anaerobic soils that are amended with glucose and water has been extended to soils from diverse environments, and having different physical and chemical properties (table 1). The results of these experiments, given in table 2, show a large variation in the number and amounts of products detected. The greatest diversity and quantity of products was found with the Honeoye silt loam, which was the best agricultural soil of those tested. Of the metabolites detected, only acetaldehyde, from the Croghan and Puerto Rico soils, and dimethylsulfide, from the Puerto Rico soil, were not found with the Honeoye soil. With the exception of dimethyl sulfide, all the metabolites detected were typical glucose fermentation products. Dimethyl sulfide was presumed to be derived from the native organic matter in the Puerto Rican soil sample.

Volatile products were not detected from the Antartica soil samples, which is consistent with Camerons report (10) that these samples did not contain anaerobes, or from the Salt Lake soil.

b) Soils amended with glucose plus amino acids

In preliminary experiments volatile organic products were not detected when sodium glutamate, methionine , phenylalanine, isoleucine and threonine were incubated individually, or as a mixture, with Honeoye silt loam (1970-71 report). Apart from glutamate, the amino acids used have been classified by Greenwood and Lees (1) as being resistant to anaerobic breakdown in soil. These authors found that the resistant amino acids were metabolized at a faster rate when supplied in a complex mixture of amino acids (casein) and concluded that "the microorganisms effecting anaerobic breakdown of some of the common amino acids may require a complex mixture

of substances for growth". In our study, Bacto-Peptone was supplied as a complex amino acid mixture, but volatile products were not detected during the 50 days of the experiment. In an attempt to induce utilization of amino acids, Honeoye silt loam samples were amended with glucose plus sodium glutamate and glucose plus peptone. The only difference between the glucose and glutamate amendment and glucose alone was the detection of a small quantity of acetaldehyde and a shift in the period of maximal metabolite production from the second and third weeks to the first and second weeks (cf.figs. 1 and 2). Only small amounts of volatile products were detected when the Honeoye soil was amended with glucose and peptone (figure 3) but two sulfur containing compounds (dimethyl sulfide and dimethyldisulfide), which are probably derived from the sulfur containing amino acids present in peptone, were found.

To find which of the sulfur containing amino acids in peptone were being utilized, the Honeoye silt loam was amended with glucose together with either cystine, cysteine or methionine. Cystine almost completely inhibited substrate metabolism, only small amounts (<20 µg) of acetone and methyl ethyl ketone being detected. Cysteine also had an adverse effect on substrate metabolism (cf. figs. 1 and 4) but not to the same extent as cystine. Volatile sulfur compounds were not detected in either of these amendments although it appeared that metal sulfides were being formed in the soil since it turned black after one week with cystine and after two weeks with cysteine. This observation is consistent with the known anaerobic catabolism of cystine and cysteine (2). Indeed, apart from one questionable report (3), volatile organic sulfur compounds have not been detected from either aerobic or anaerobic catabolism of cystine or cysteine; sulfate or sulfide, respectively, being the usual products (2). Volatile thiols and organic sulfides are, however, commonly reported products of methionine decomposition, under both aerobic and anaerobic conditions (2). When Honeoye silt loam was amended with glucose and methionine, the usual glucose products were detected together with methane thiol, dimethylsulfide, dimethyl disulfide, and a 4-carbon compound tentatively identified as 2-methyl propane thiol (figure 5). It is not certain that dimethyl disulfide was a biological product since this compound was formed by chemical oxidation of methane thiol during g.c.-m.s. analysis of authentic samples of methane thiol.

Other soils were amended with glucose and methionine and glucose and cysteine to determine whether the response would be similar to that observed with the Honeoye soil. The four volatile organic sulfur compounds were detected when the Puerto Rico soil was amended with glucose and methionine (figure 6) and the total quantity of metabolites was increased (cf. figs. 6 and 8). Addition of methionine appeared to inhibit utilization of glucose in the Croghan soil since the only product detected was 1-butanol.

When glucose and cysteine were added to the Puerto Rico soil, the quantity of products was much reduced compared to amendment with glucose alone (cf. figs. 7 and 8) and no volatile sulfur products were detected. The response was quite similar to that obtained with the Honeoye soil. In contrast, the quantity of products generated increased markedly when the Croghan soil was similarly amended compared to addition of glucose alone (cf. figs 10 and 11). The major difference in products between the two

amendments was the formation of large amounts of butyric and acetic acids when cysteine was present, whereas with glucose alone, a small amount of butyric acid was detected and no acetic acid. Diacetyl was another product detected only in the glucose and cysteine amendment. Formation of butyric acid was also noticed when cysteine was present in the Puerto Rico and Honeoye soils, even though the overall production of volatile organic metabolites was reduced.

Ethanol, which was the sole product detected when glucose was added to the Fresno soil, was generated in larger amounts when glucose and cysteine were added (cf. figs. 11 and 12), and two other products, acetaldehyde and propanol were detected.

Although the effect of adding cysteine together with glucose (compared to glucose alone) on the quantity of volatile organic products generated was not predictable, there appeared to be a qualitative trend towards acid production, i.e. from ethanol to acetic acid and butanol to butyric acid, in some of the soils tested.

From our studies and published literature (4-7), it would appear that volatile organic sulfur compounds formed in terrestrial soils are not derived from cysteine or cystine, but may be derived from methionine. Barrows has reported (8) that the fate of methionine sulfur is influenced by the presence of cysteine, with increasing amounts of methionine sulfur being converted to sulfate as the ratio of methionine:cysteine decreases. The fate of sulfur in organic energy sources may therefore be dependent on what sulfur containing compounds are being utilized.

c) Soils amended with amino acid mixture following pre-incubation with carbon source

Volatile amines (or ammonia) had not been detected in any of the experiments where amino acids were present in the amendment. Amine formation by decarboxylation of amino acids requires a pH less than 5.5, and in most cases less than 4.5 (9) so it is possible that the pH of the soil was the limiting factor. Since incubation of the soils with substrate usually lowered the pH by approximately one unit an amino acid mixture (lysine, ornithine, histidine, tyrosine and glutamic acid) was added to soils that had been pre-incubated with substrate. The addition was made 50 days after the initial amendment, at which time volatile products were not being generated. The amino acid mixture was added to the Croghan soil after it had been incubated with glucose and cysteine, and to the Puerto Rico soil after it had been incubated with glucose alone, glucose and cysteine or glucose and methionine. Products were detected for as long as four weeks following addition of the amino acid mixture but no amines (or ammonia) were detected (table 3). Trace amounts of acetone and MEK, and an estimated 100 µg of benzene were the only products detected with the Croghan soil. Although benzene could have been derived from tyrosine, its occurence as an artifact has not been ruled out. Ketones and alcohols were the dominant products in the Puerto Rico soil samples regardless of the pre-treatment. Butyric acid formation was noted where methionine or cysteine had been present in the initial amendment and volatile sulfur compounds were detected when glucose or glucose and methionine

had been the original amendment. Benzene (~ 10 µg) was detected in one sample after two weeks incubation with the amino acid mixture, but again it is not certain that it is a biological product. Experiments with soils amended with glucose and tyrosine are currently being conducted in an attempt to generate benzene.

Effect of addition of carbon source

None of the soils that were amended with carbon source and water yielded volatile organic products when amended with water alone. The effect of adding carbon source(s) on the generation of volatile organic products is summarized in the following statements:

1. The response to addition of a single substrate varies with the soil e.g. variation with glucose as substrate.

2. The response with a given soil varies with substrate e.g. glucose vs. amino acids, fatty acids, purines and various sulfur compounds added to Honeoye silt loam (see also 1970-71 report).

3. For a given soil, addition of more than one substrate may be beneficial or detrimental e.g. Puerto Rico clay and glucose (norm) vs. glucose and methionine (beneficial) vs. glucose and cysteine (detrimental).

4. Soils may respond differently to addition of more than one substrate cf. Honeoye silt loam and glucose (norm) vs. glucose and cysteine (detrimental) and Croghan sandy loam and glucose (norm) vs. glucose and cysteine (beneficial).

These statements reflect the fact that metabolism of substrate and generation of volatile products are dependent on the presence (or absence) of particular microbial communities in a soil sample, and on the physical and chemical environment that the soil provides.

IN SITU STUDIES

Following development of a reliable pumping system, studies were conducted at two locations:

1. The lycopodium site described in the 1970-71 report

2. The edge of Cornell vegetable crops field, in tilled soil 4 ft. from growing corn.

At the lycopodium site, the probe was inserted 3" into the 6" core and the soil atmosphere was pumped through the poropak trap at 2 ml/min. The trap was analyzed after one week, and small quantities of nonan-2-one and heptan-2-one were detected. The results were similar to those previously reported, and did not vary during the two month period over which the experiments were carried out.

At the Vegetable Crops site, cores were taken 6" and 18" deep and the probe inserted 3" and 6" respectively.

The soil atmosphere was sampled at 5 ml/min. for one week at which time carbon dioxide, butene (? of 1- or 2-), t-butanol, 1-butanol, butyric acid, and one unidentified peak were detected in small quantities ($\sim$ 5 µg per compound) in both experiments.

The in situ experiments yielded little in the way of organic metabolite which may be due to the following reasons:

1. Only small quantities of organic products being generated in the essentially aerobic soil system. (Possibly a factor since carbon dioxide, which is only trapped poorly, was detected.)

2. An inefficient collection system - it is not known how much of the gas that is being pumped actually comes from the soil, or whether that which is being removed is replaced by the soil or above ground atmosphere.

The efficiency of the collection system used and the others described in the 1972-73 research proposal is being evaluated using soil samples contained in drums, and modified with carbon source. These experiments will also give an indication of the volume of soil that is being sampled by the various procedures, which is important since one advantage of an in situ procedure could be a large sample size.

SENSITIVITY OF THE METHOD

a) To Organisms

Honeoye silt loam was amended with glucose in the usual manner and incubated under argon for seven days, close to the minimum period for detection of products. After seven days, the quantity of organic products accumulated and the microbial population were measured.

Product	Quantity (μg) at day 7	Minimum detectable level (μg)	Number of cells
Ethanol	914	1.25	
Acetone	51	0.75	
Isopropanol	98	0.75	3.5×10^6
n-propanol	62	0.75	
2-butanol	7	0.5	
i-butanol	17	0.5	
1-butanol	60	0.5	

Thus for the major metabolite, ethanol, the minimum detectable number of cells in our system for the example given is

$$\frac{1.25}{914} \times 3.5 \times 10^6 \simeq 5 \times 10^3$$

The sensitivity of the method is presently being determined in a similar way but using a sterile soil sample that has been innoculated with a known number of cells of a single species, isolated from previous experiments.

b) Volatile Organic Products vs. CO_2

Measurement of sensitivity of a life detection method is dependent on the sensitivity of the analytical instrument or method used so that absolute numbers may not be very meaningful. A more realistic evaluation of the method described can be obtained by comparing the % of added carbon which is released as CO_2 versus that which is released as volatile organic products. For the Honeoye soil, amended with glucose and incubated in an anaerobic environment, 7.5-10% of the added carbon is released as volatile organic compounds (Table 2) and 20-25% as CO_2. If the carbon released as organic compounds is equally divided between four major products (varied from 1 to 4 in our experiments), the amount of carbon released in each of the four compounds

is approximately one order of magnitude less than that released as CO_2. If gas chromatography, or combined g.c.-m.s., is the analytical system used, it is quite possible that the sensitivity of detection for CO_2 would be an order of magnitude less than that for the organic products, and so a smaller number of cells could be detected by monitoring the production of individual organic compounds rather than CO_2.

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TABLE I

Chemical Analysis of Soils

					Alkali Soils			Antartica soils*	
	Honeoye silt loam	Croghan sandy loam	Puerto Rico clay	Arizona sand	Fresno (Cal.)	Death Valley (Cal.)	Salt Lake (Utah)	#500 loamy sand	#667 sand
Organic Carbon (%)	1.8	2.7	3.2	0.3	0.4	0.3	0.2	0.09	0.02
P (ppm)	10	<0.5	<0.5	7.5	140	23	2.5	-	-
K (ppm)	68	15	450	130	300	300	1000	5	-
Mg (ppm)	225	38	225	153	200	358	675	71	-
Ca (ppm)	3700	1250	1000	600	78	18,875	10,000	190	-
Mn (ppm)	17	2	94	40	1	23	1.5	-	-
Fe (ppm)	0.5	10	2.5	0.5	1	1	<0.5	-	-
Al (ppm)	5	160	30	12.5	175	5	1	-	-
NO_3^- N ppm	125	5	48	<2.5	30	5	1	-	-
NH_3 N ppm	2.5	8	6	8.5	7.5	1	1	-	-
Total N (%)	0.176	-	0.39	0.028	-	0.012	0.008	0.007 (org. N)	0.002 (org. N)
pH	6.5	5.9	5.3	5.9	9.6	8.7	9.2	8.0	7.5

*Obtained from R. E. Cameron, Jet Propulsion Laboratory. Analysis for Sample #500 taken from Cameron, 1971, J.P.L. Tech. Report 32-1522, p5 and for sample #667 from Cameron et al., 1970, Antartic Ecology 2:702-716. Blank spaces indicate measurements not determined.

TABLE II

Generation of Volatile Organic Products in Soils Amended with Glucose

Product (μg)	N_2	Ar^b				
	Honeoye[a]	Honeoye[a] & $MgSO_4$	Honeoye[a]	Arizona	Puerto Rico[a]	Croghan
Ethanol	420	2360	1500	5	110	230
n-Propanol	40	430	520			15
i-Propanol	125	845	850	45		
n-Butanol	4000	985	820	3025	225	190
2-Butanol	15	280	140			
i-Butanol	5	25	65			
Butyric Acid	670					
Ethyl Acetate	5	15	5			10
Butyl Acetate	5	10	40	35		
Ethyl Butyrate	180	15	15		5	40
Butyl Butyrate	430	35		10	2	25
Acetone	550	380	625	170	80	25
MEK	25	420	300	2	10	25
% C recovered as organics	10.5	8.5	7.5	5	0.7	0.9

(Continued)

Table II - Generation of Volatile Organic
Products in Soils Amended with Glucose (Continued)

[a] The following compounds were found in amounts not exceeding 15 µg

Honeoye (N_2) : propene, methanol, butyraldehyde, n-&i-propyl butyrate, 2 butyl butyrate, MPK

Honeoye (Ar) : i-amyl alcohol, ethyl crotonate, MPK

Honeoye (Ar & $MgSO_4$) : i-amyl alcohol, 2-pentanol, ethyl propionate, crotonate and i-butyrate, n-&i-propyl crotonate, i-propyl butyrate, 2-butyl butyrate, 1-&2-butyl crotonate, MPK

Puerto Rico : acetaldehyde

[b] Ethanol was the only organic product detected from the Death Valley and Fresno soils (30 and 17 ug, respectively). No organic products were detected with the Salt Lake soil or either of the soils from Antartica.

TABLE III

Effect of addition of mixture of amino acids* to soils that had been previously incubated with carbon source.

Soil	Week 1	2	3	4
Croghan sandy loam	Acetone Benzene	Acetone MEK	Benzene	------
Puerto Rico soil				
a)	Acetone Isopropanol MEK MPK	Acetone Isopropanol Dimethylsulfide	Ethanol Acetone Isopropanol MPK	Ethanol Acetone Isopropanol
b)	Acetone Isopropanol MEK MPK Butyric acid	Acetone Isopropanol MEK 1 Butanol MPK Butyric acid	Acetone	------
c)	Acetone MEK MPK Dimethyldisulfide Butyric acid	Acetone MEK Benzene Butyric acid	Acetone MEK Dimethyldisulfide	Acetone Dimethylsulfide Dimethyldisulfide

Croghan soil (10 g) received 1% glucose, 0.5% cysteine and 30% H_2O and was incubated under a stream of argon for 50 days, then an aqueous solution (1 ml) of the amino acid mixture was added and the incubation continued.

Puerto Rico soil:

a: 10 g of soil + 1% glucose + 40% H_2O incubated for 50 days under a stream of argon, then the amino acid mixture was added and the incubation continued.

b: Same as 'a' but initial amendment included 0.5% cysteine.

c: Same as 'a' but initial amendment included 0.5% methionine.

* 100 mg of a mixture containing equal weights of lysine, ornithine, histidine, tyrosine and glutamic acid.

KEY TO FIGURES 1-12

A	Water
B	Acetaldehyde
C	Methane thiol
D	Ethanol
E	Acetone
F	i-Propanol
G	Dimethyl sulfide
H	n-Propanol
I	Acetic acid
J	Diacetyl
K	Methyl ethyl ketone (Butan-2-one)
L	Ethyl acetate
M	2-Butanol
N	i-Butanol
O	n-Butanol
P	2-methyl propane-1-thiol
Q	Methyl propyl ketone (Pentan-2-one)
R	Dimethyl disulfide
S	Butyric acid
T	i-amyl alcohol
U	Ethyl butyrate
V	n-Butyl acetate
W	Ethyl crotonate
X	n-Butyl butyrate

Unlettered peaks are unidentified compounds.

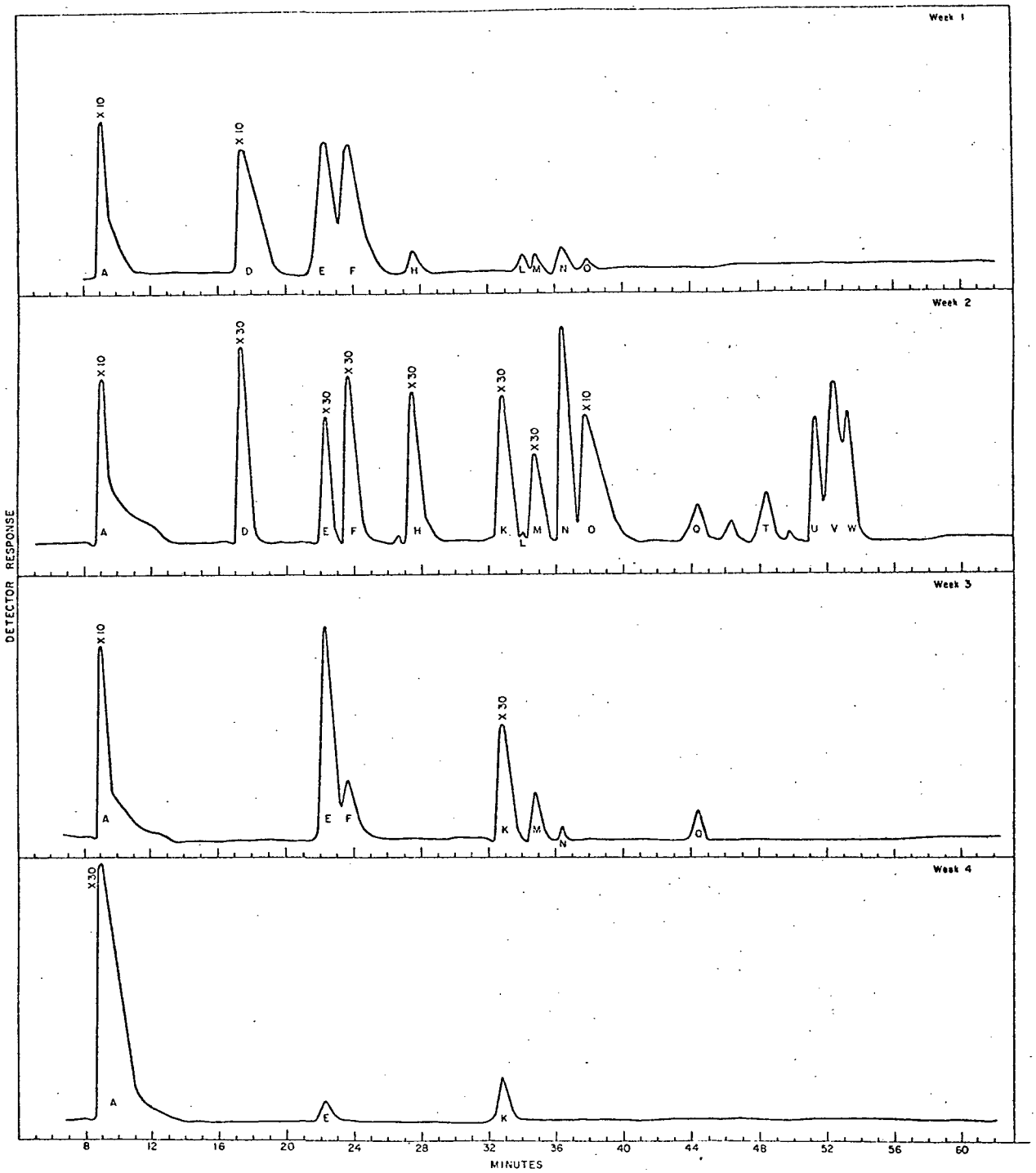

FIG. 1.--Products generated in Honeoye silt loam amended with glucose (1% w/w). Analysis on Poropak QS held at 25° for 4 min. then programmed to 100° at 32°/min, from 100-130° at 10°/min, 130-190° at 2°/min and 190-250° at 5°/min. Attenuation of unmarked peaks is X100, the most sensitive setting.

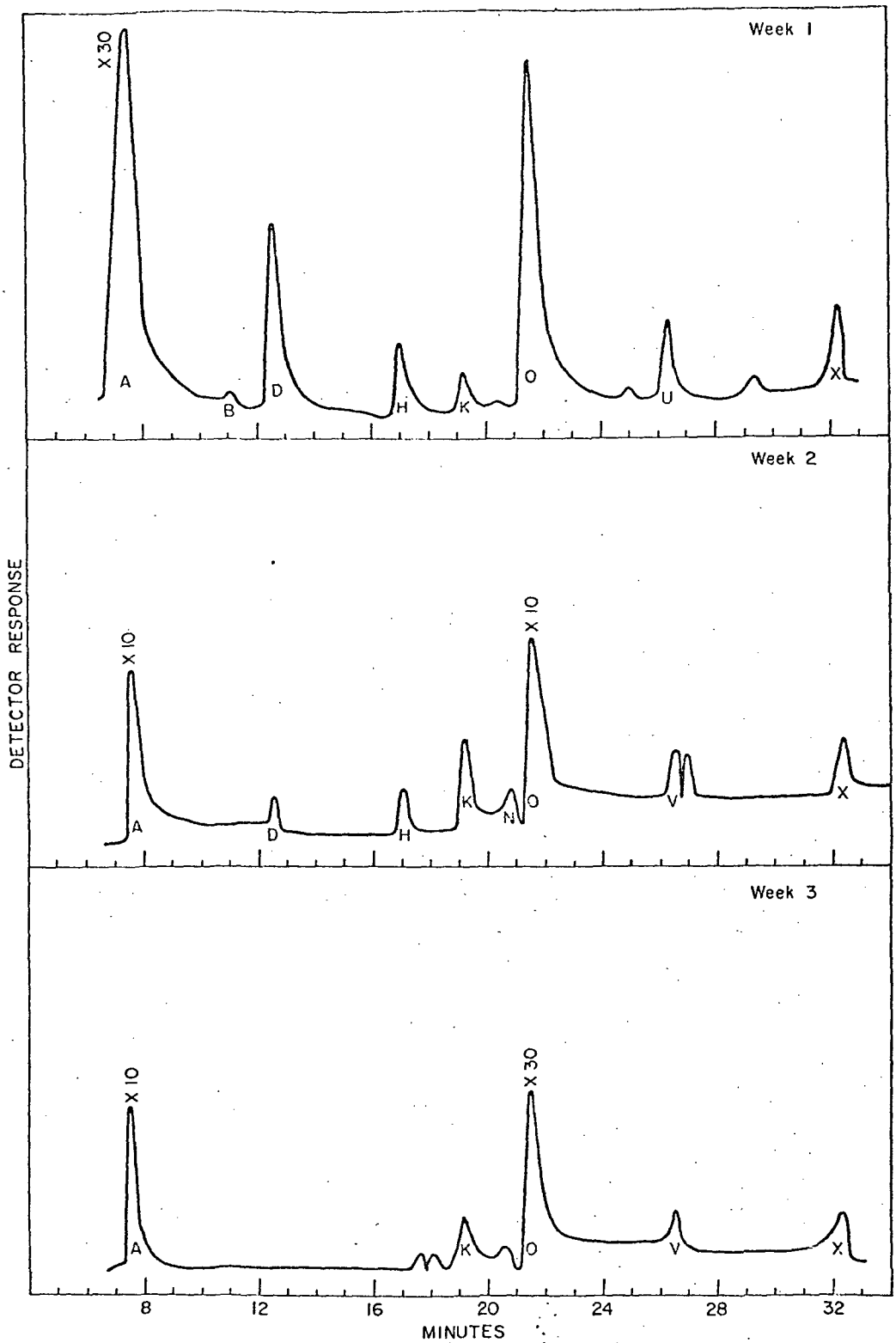

FIG.2.--Products generated in Honeoye soil amended with glucose (1% w/w) and sodium glutamate (0.5% w/w). Analysis on Chromosorb 101 held at 25° for 4 min. then programmed to 100° at 32°/min. and from 100 to 250° at 5°/min. Attenuation of unmarked peaks is X100, the most sensitive setting.

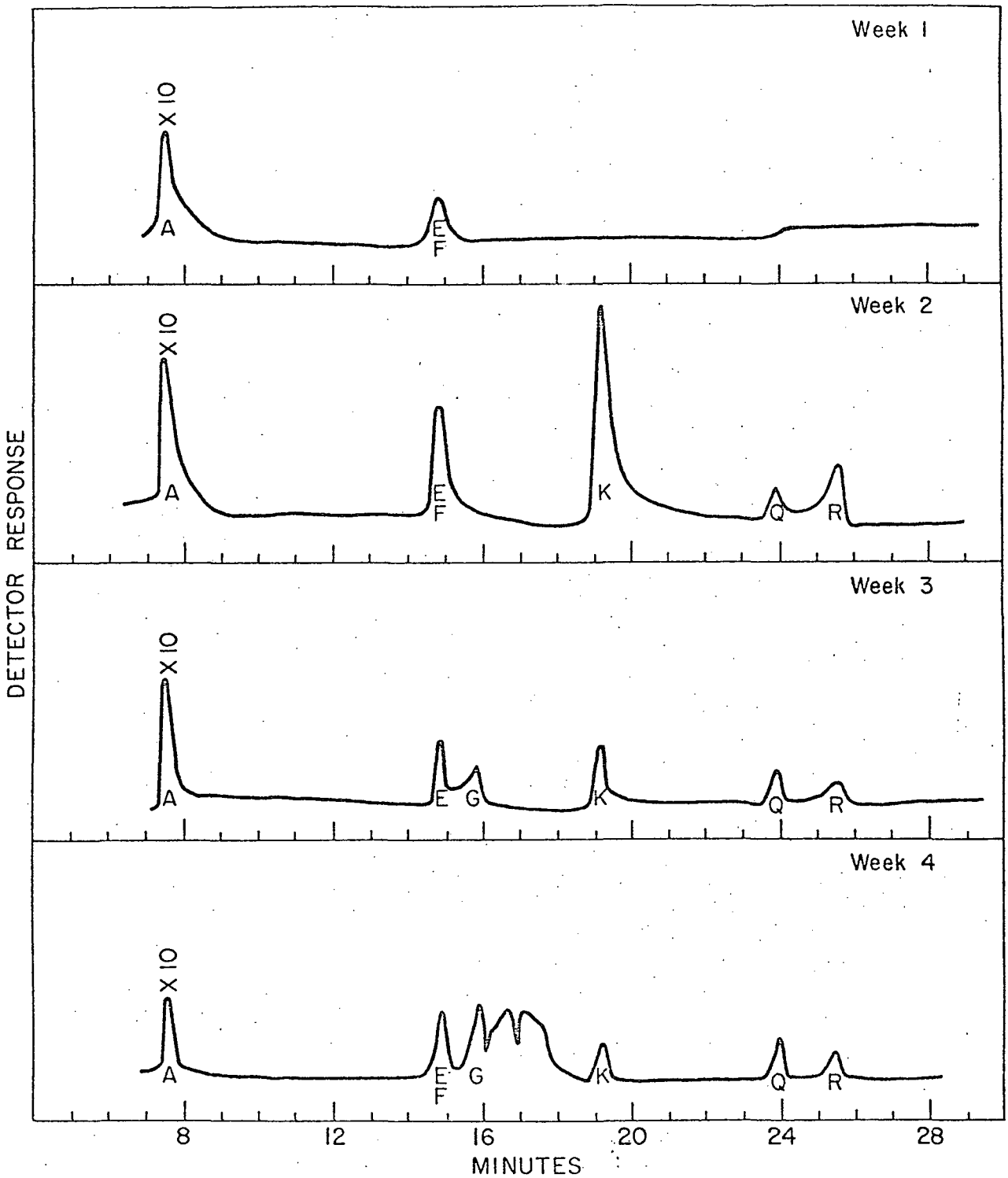

FIG.3.--Products generated in Honeoye soil amended with glucose (1% w/w) and peptone (0.5% w/w). Analysis on Chromosorb 101 held at 25° for 4 min. then programmed to 100° at 32°/min. and from 100 to 250° at 5°/min. Attenuation of unmarked peaks is X100, the most sensitive setting.

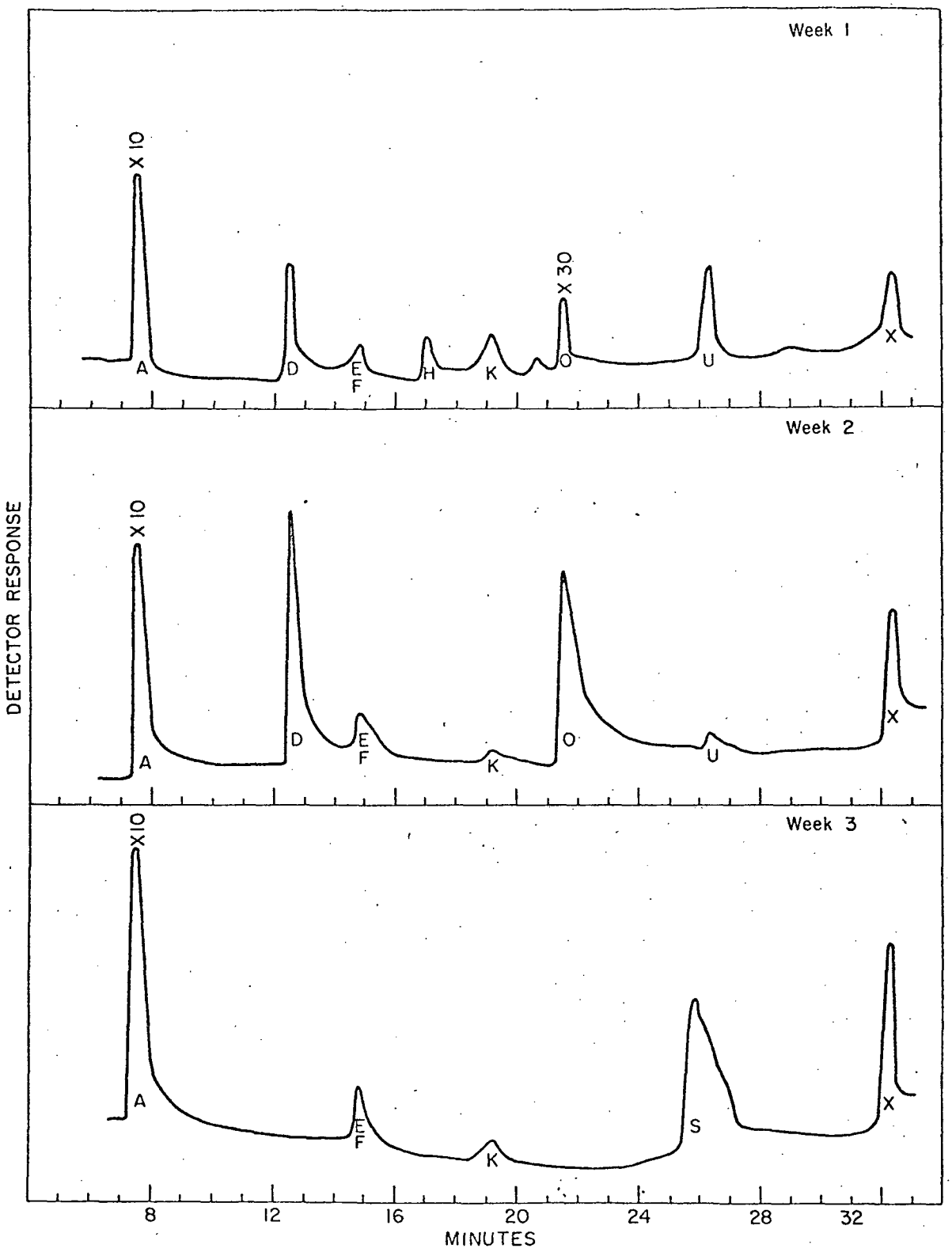

FIG. 4.--Products generated in Honeoye soil amended with glucose (1% w/w) and cysteine (0.5% w/w). Analysis on Chromosorb 101 held 4 min. at 25° then programmed to 100° at 32°/min. and from 100 to 250° at 5°/min. Attenuation of unmarked peaks is X100, the most sensitive setting.

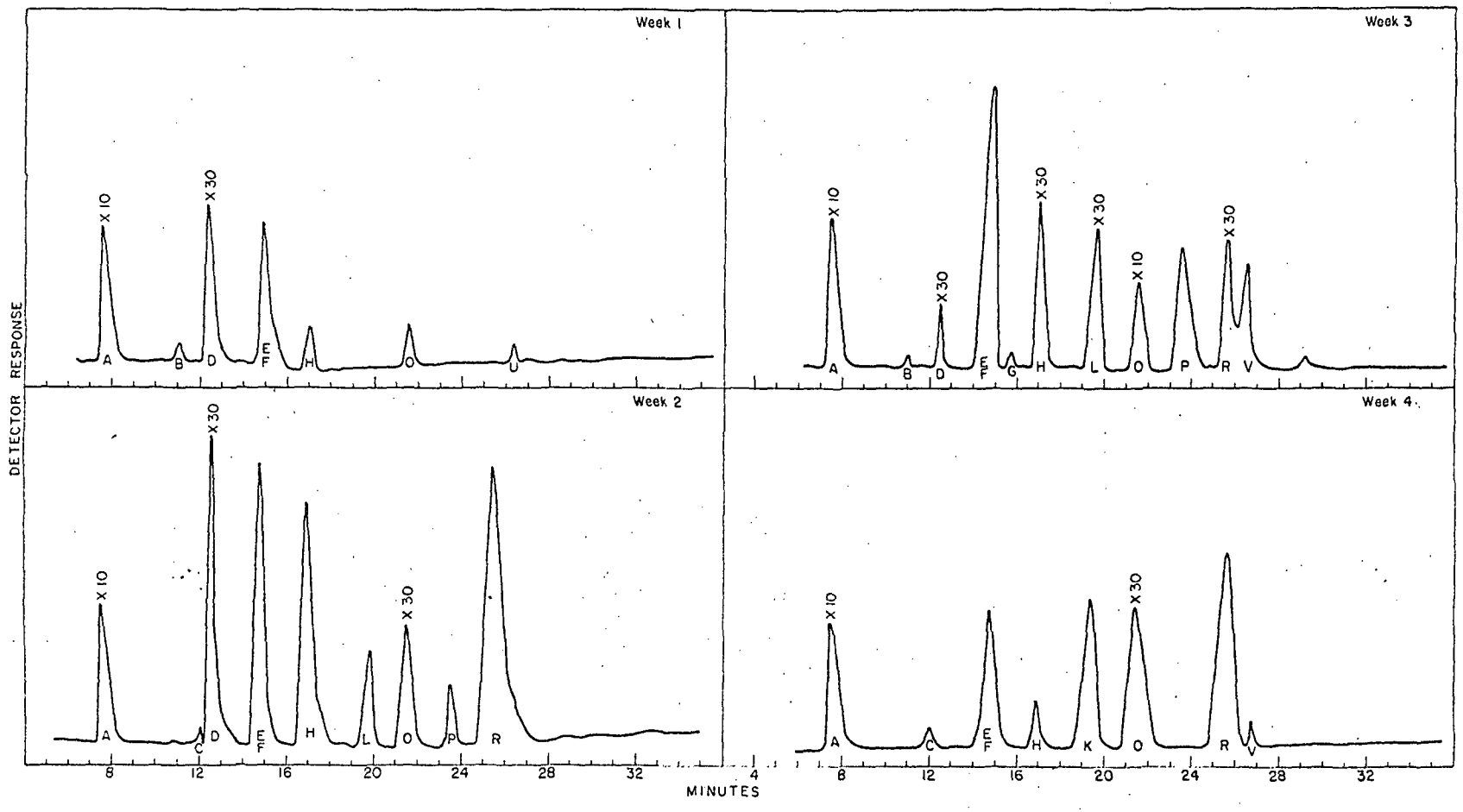

FIG. 5.--Products generated in Honeoye soil amended with glucose (1% w/w) and methionine (0.5% w/w). Analysis on Chromosorb 101 held 4 minutes at 25° then programmed to 100° at 32°/min. and from 100 to 250° at 5°/min. Attenuation of unmarked peaks was X100, the most sensitive setting.

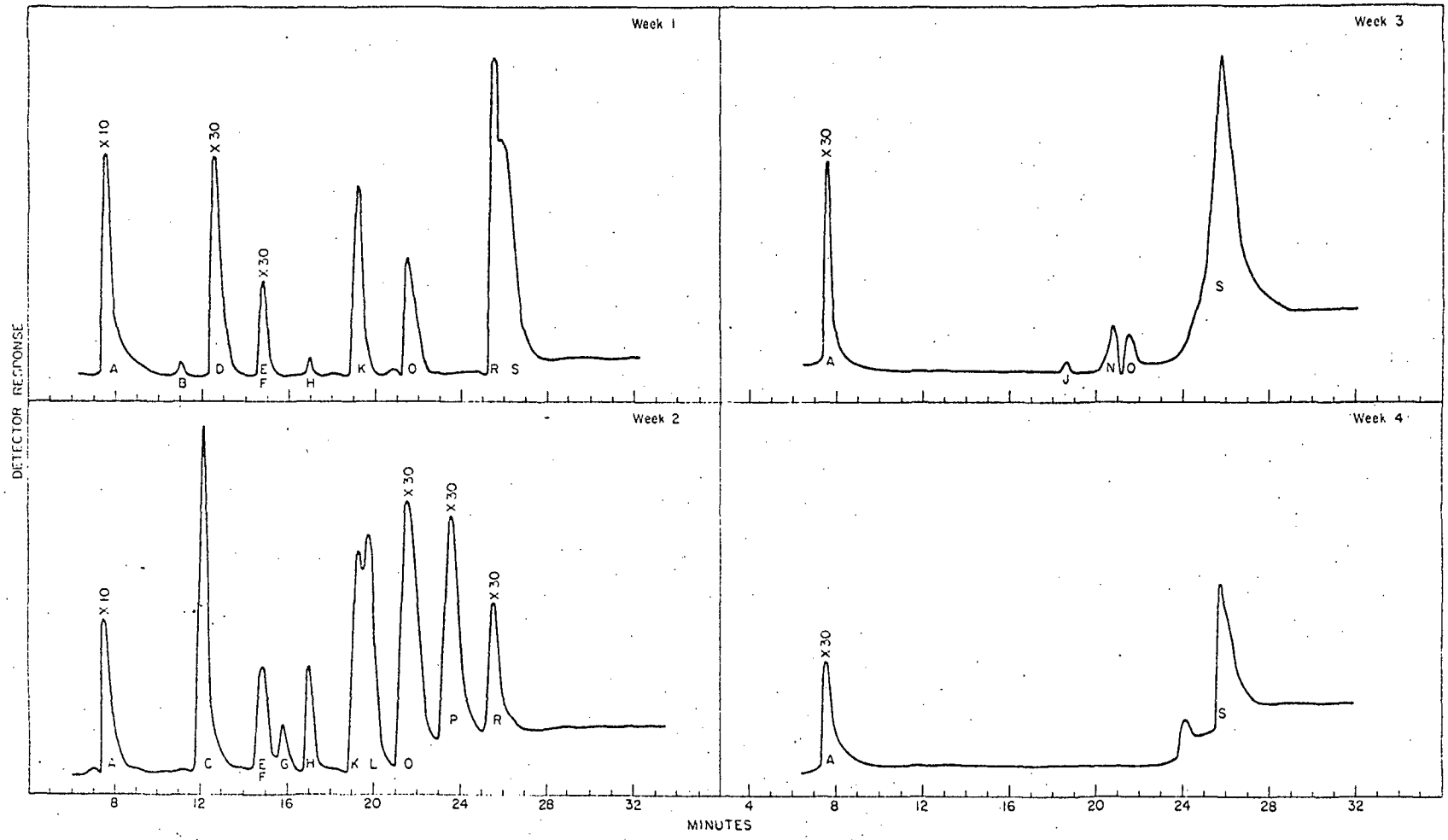

FIG. 6.--Products generated in Puerto Rico soil amended with glucose (1% w/w) and methionine (0.5% w/w). Analysis on Chromosorb 101 held at 25° for 4 min. then programmed to 100° at 32°/min. and from 100 to 250° at 5°/min. Attenuation of unmarked peaks is X100, the most sensitive setting.

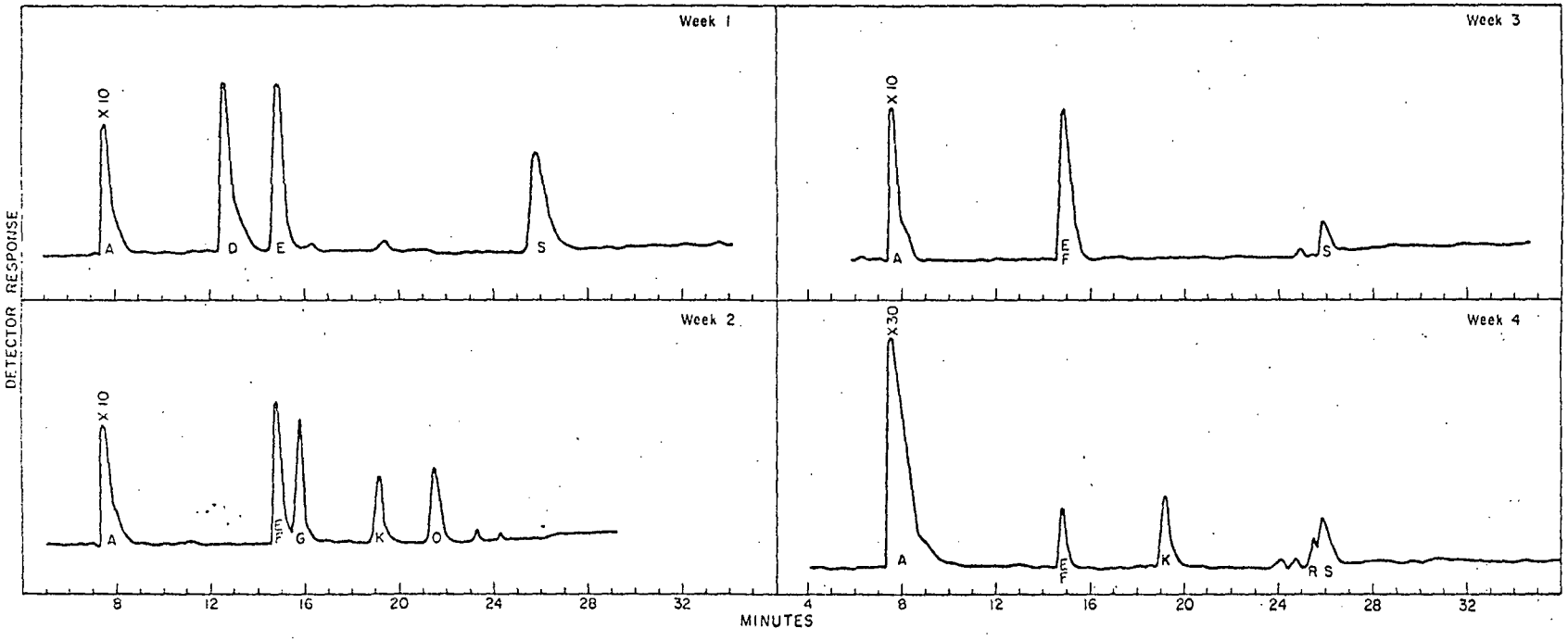

FIG. 7.--Products generated in Puerto Rico soil amended with glucose (1% w/w) and cysteine (0.5% w/w). Analysis on Chromosorb 101 held at 25° for 4 minutes, then programmed to 100° at 32°/min. and from 100 to 250° at 5°/min. Attenuation of unmarked peaks is X100, the most sensitive setting.

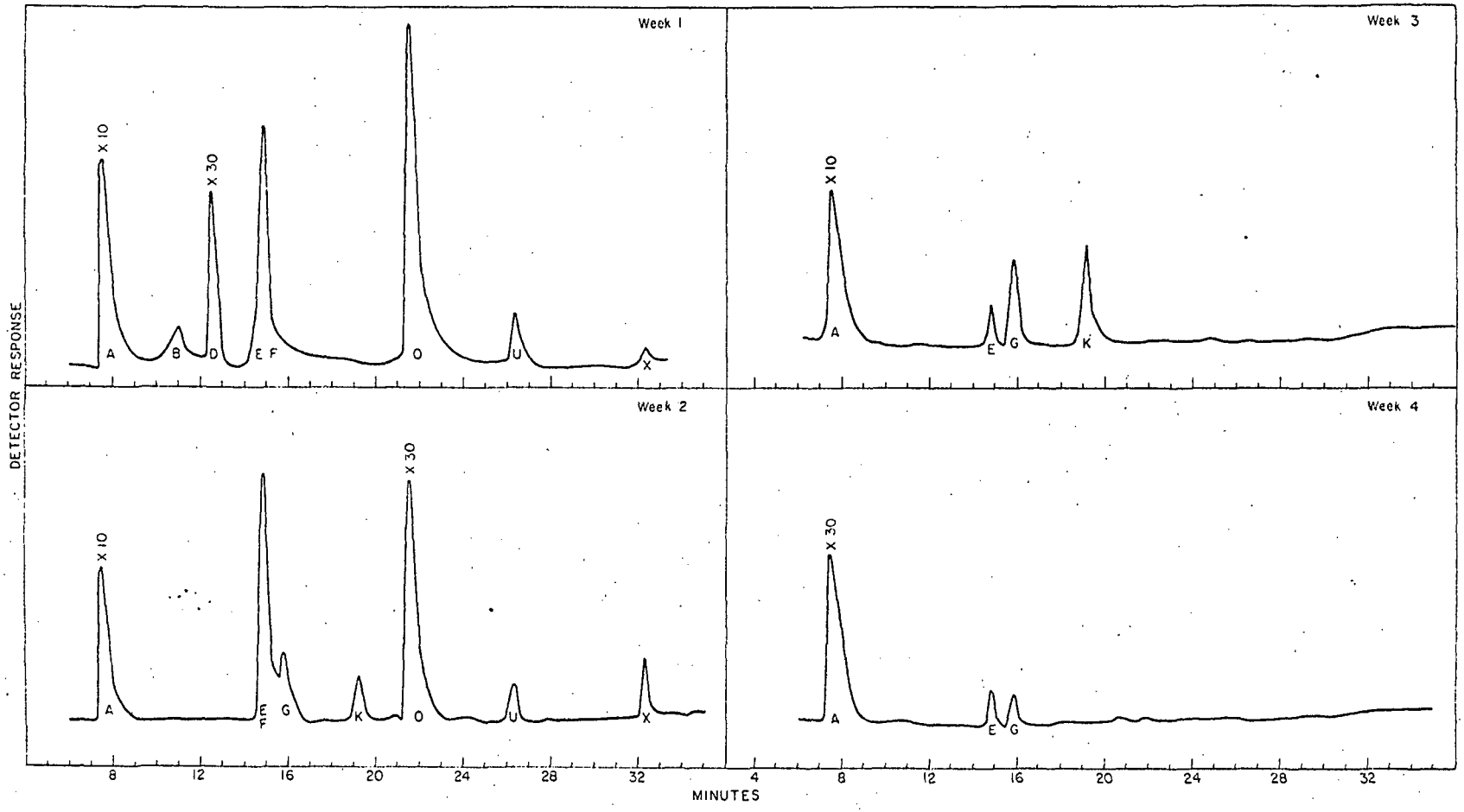

FIG. 8.--Products generated in Puerto Rico soil amended with glucose (1% w/w). Analysis on Chromosorb 101 held at 25° for 4 min. then programmed to 100° at 32°/min. and from 100 to 250° at 5°/min. Attenuation of unmarked peaks is X100, the most sensitive setting.

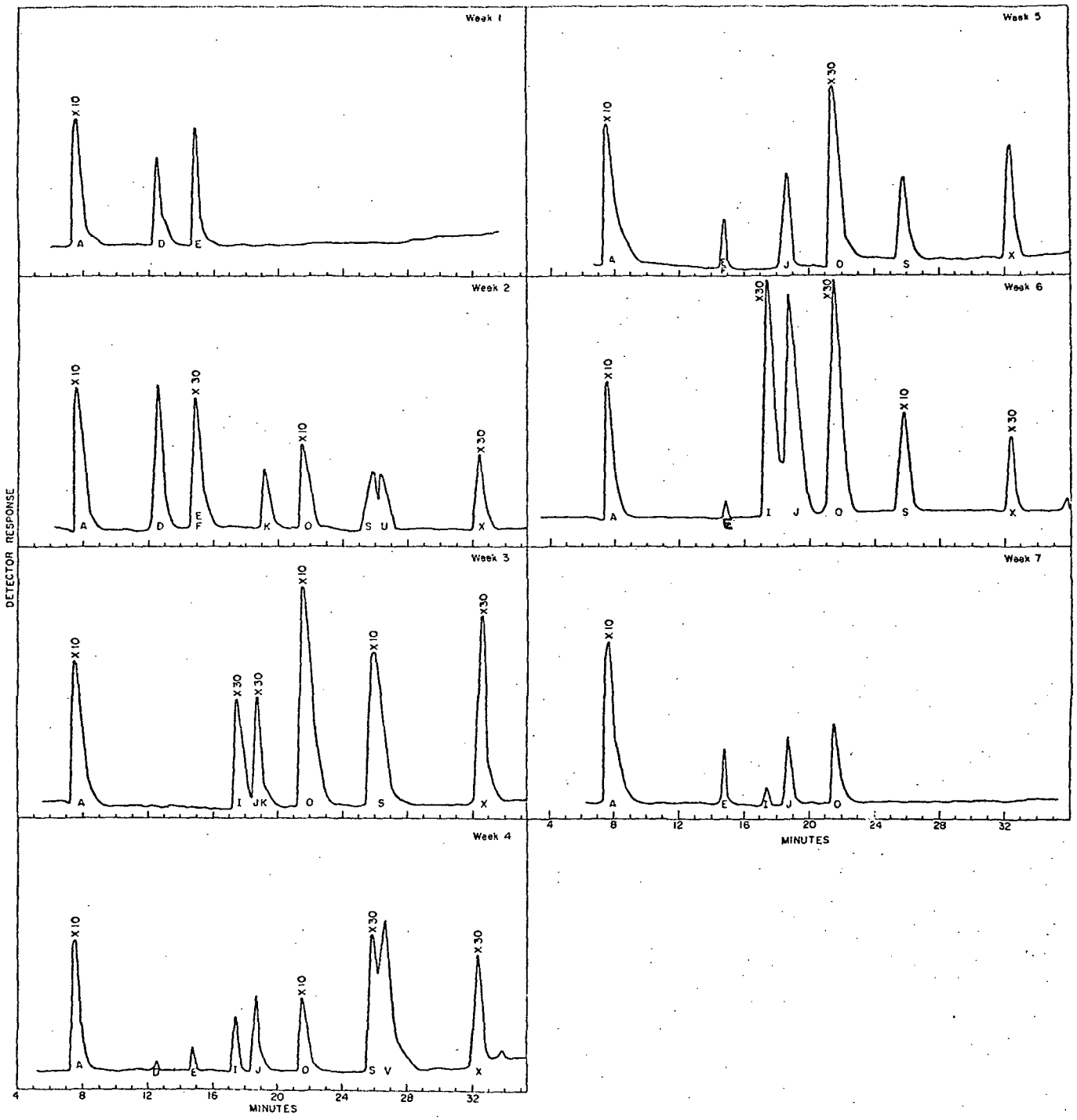

FIG. 9.--Products generated in Croghan soil amended with glucose (1% w/w) and cysteine (0.5% w/w). Analysis on Chromosorb 101 held at 25° for 4 minutes then programmed to 100° at 32°/min. and from 100 to 250° at 5°/min. Attenuation of unmarked peaks is X100, the most sensitive setting.

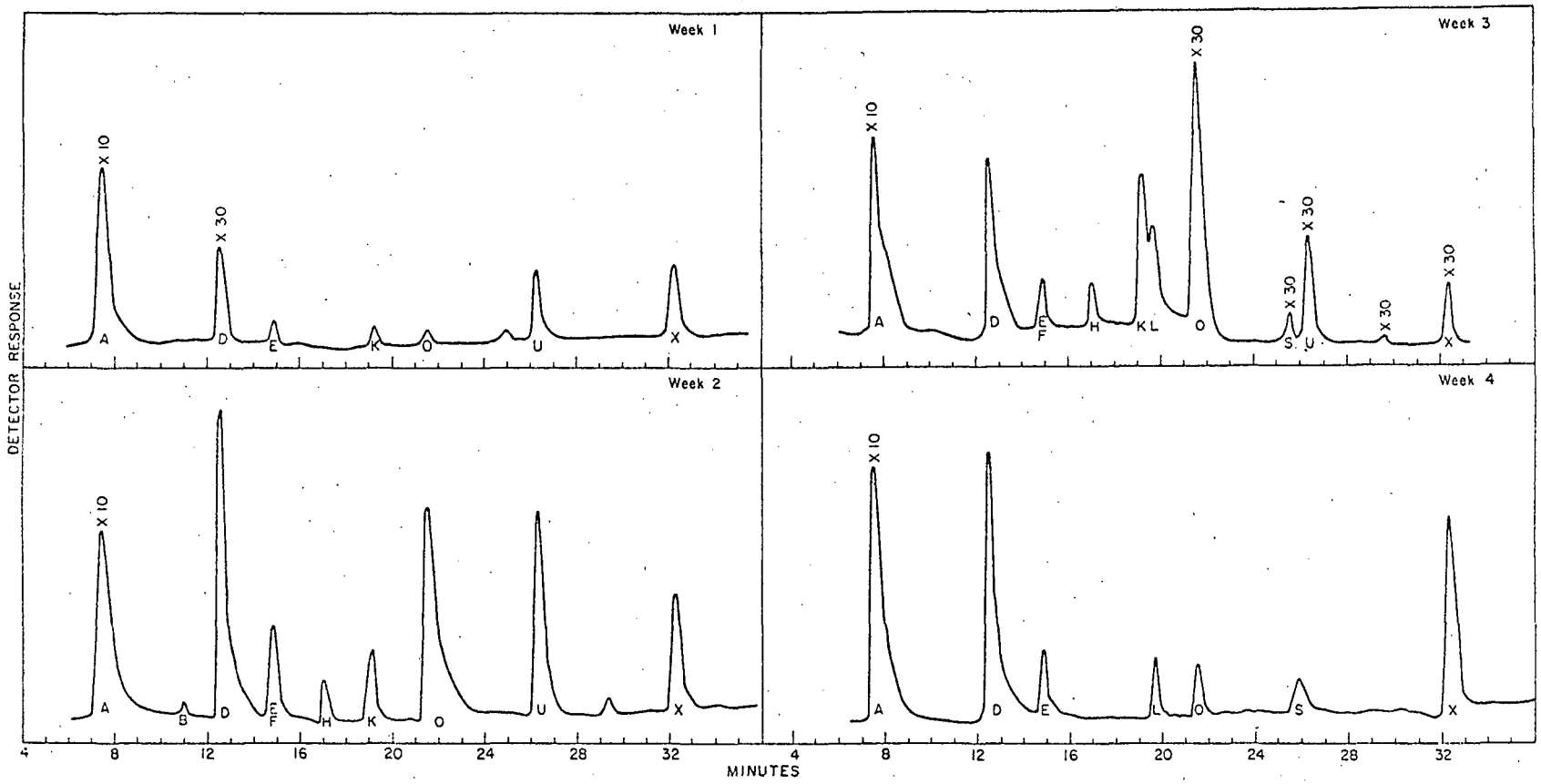

FIG. 10.--Products generated in Croghan soil amended with glucose (1% w/w). Analysis on Chromosorb 101 held at 25° for 4 min. then programmed to 100° at 32°/min. and from 100 to 250° at 5°/min. Attenuation of unmarked peaks is X100, the most sensitive setting.

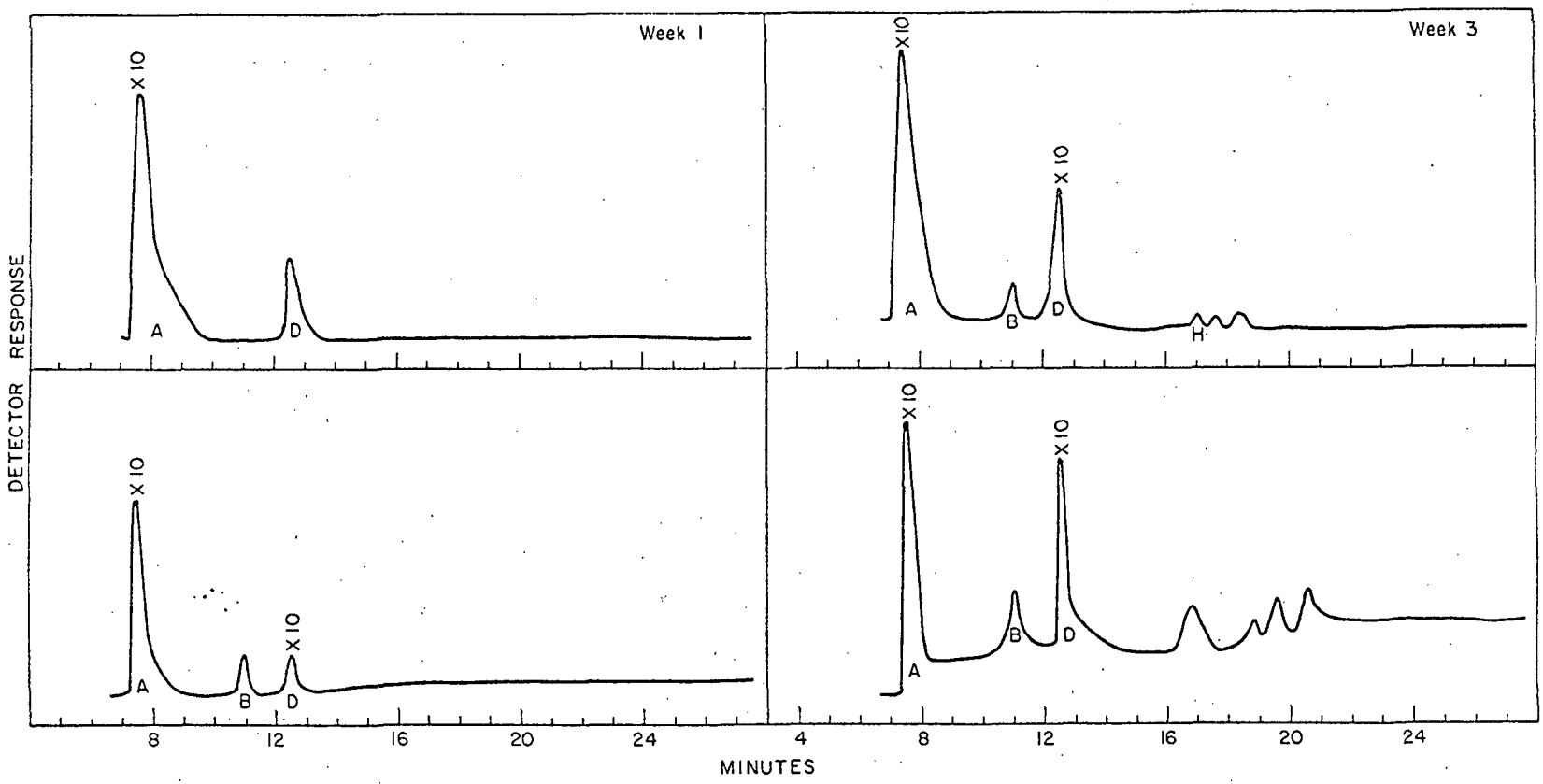

FIG. 11.--Products generated in Fresno soil amended with glucose (1% w/w) and cysteine (0.5% w/w). Analysis on Chromosorb 101 held at 25° for 4 minutes then programmed to 100° at 32°/min. and from 100 to 250° at 5°/min. Attenuation of unmarked peaks is X100, the most sensitive setting.

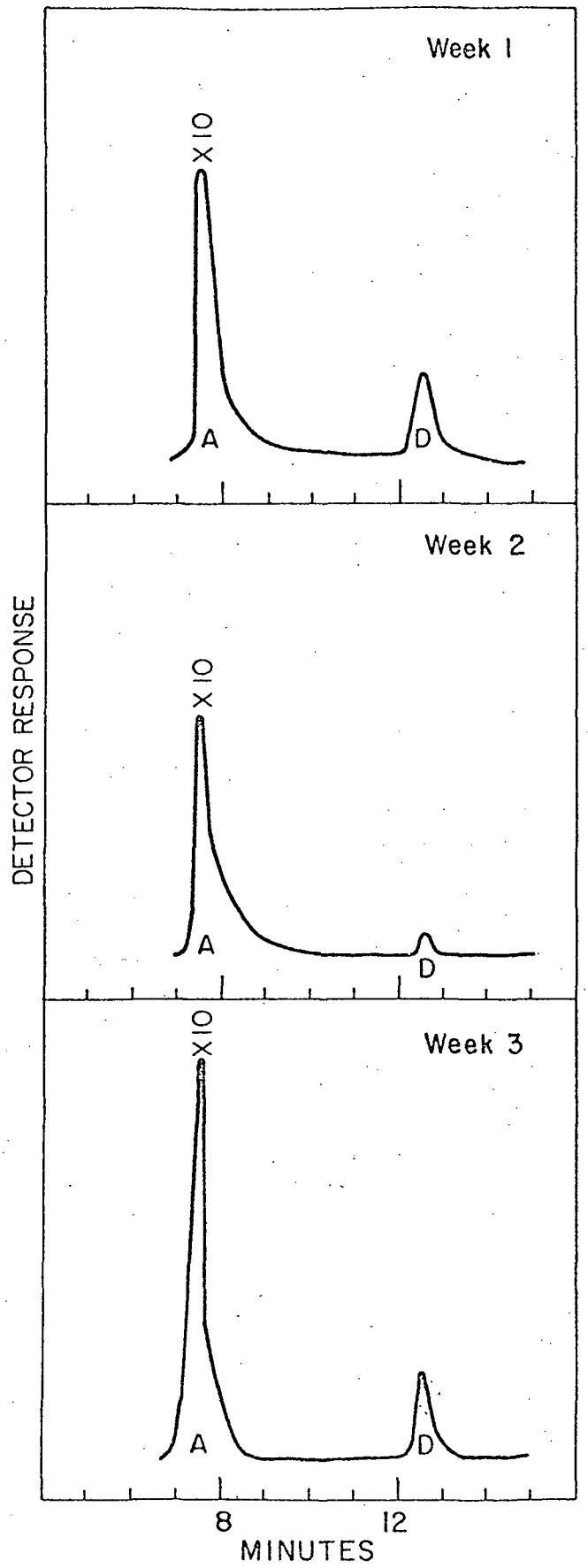

FIG. 12.--Products generated in Fresno soil amended with glucose (1% w/w). Analysis on Chromosorb 101 held at 25° for 4 minutes, then programmed to 100° at 32°/min. and from 100 to 250° at 5°/min. Attenuation of unmarked peaks is X100, the most sensitive setting.

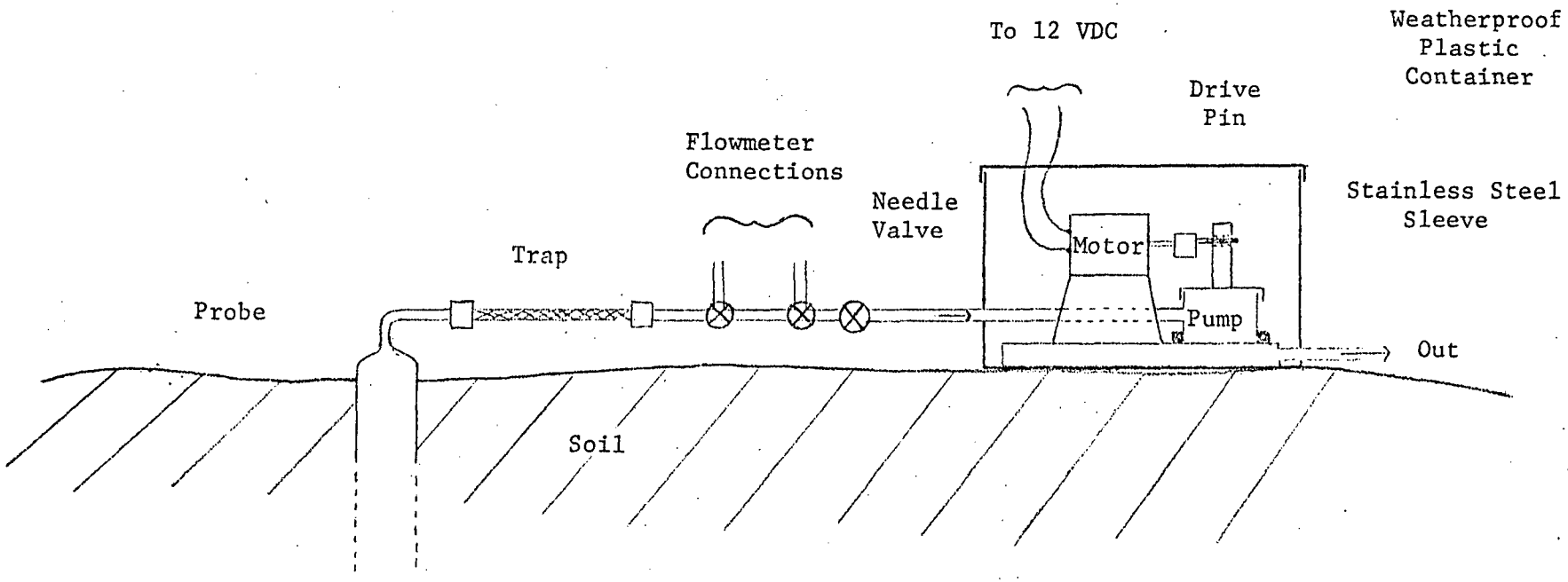

Fig. 13. In Situ Sampling System